SYMPOSIUM ON THE LACTIC ACID BACTERIA[1]

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INTRODUCTION

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The lactic acid bacteria have attracted the attention of bacteriologists since the beginning of bacteriology. They have been of particular practical interest to the food and fermentation industries. The chemical activities of certain species are essential in the manufacture of a variety of foods and fermented products, whereas certain other species ruin these products. A few species are of medical importance.

The streptococci and lactobacilli particularly have been subjected to much taxonomic and basic research. However, taxonomists recognize an increasing need for satisfactory means of identifying, differentiating, and characterizing the genera and species in the family Lactobacteriaceae.

During the past decade, special attention and investigation have been directed to the role of vitamins and amino acids in the nutrition and physiology of the lactic acid bacteria. The results of studies during this brief period have provided fundamental information of incalculable value toward improving the nutrition of higher forms of life, particularly of man. Furthermore, they show that certain selected species and strains of lactic acid bacteria are not only "indispensable analytical tools for biochemical and nutritional research," but are also extremely useful for assaying foods and other materials for their contents of vitamins and amino acids. Thus, an entirely new field of bacteriological research has developed.

Much fundamental and practical information has been obtained recently

[1] Presented at the 52nd annual meeting of the Society of American Bacteriologists at Boston, April 28, 1952.

Ralph P. Tittsler was the convenor and has served as editor of these papers.

concerning the role of certain lactic acid bacteria in the manufacture of foods and beverages, especially meat products.

The topics of the reviews presented at the *Symposium on the Lactic Acid Bacteria* were selected to provide basic and practical information on the taxonomy, nutrition, vitamin and amino acid requirements, and industrial significance of the lactic acid bacteria. Each contributor is a recognized authority in his field.

PART I. THE SYSTEMATIC RELATIONSHIPS OF THE LACTIC ACID BACTERIA[2]

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The family name Lactobacteriaceae was applied by Orla-Jensen (95) to a physiological group of gram positive rods and cocci that ferment carbohydrates either to lactic acid alone or to lactic and acetic acids, alcohol and carbon dioxide. The similarity of the lactic acid producing rods and cocci was recognized by Beijerinck (9) when he proposed the generic names *Lactobacillus* and *Lactococcus* (*Streptococcus*). Orla-Jensen (94) suggested separation of the homofermentative species from the heterofermentative types, and proposed several new generic names.

The lactic acid bacteria obtain their energy by partial fermentation of sugars without utilization of free oxygen. This necessitates utilization of considerable quantities of sugar to obtain relatively small amounts of energy for growth. At the same time, comparatively large amounts of determinable fermentation end products are produced. Each species and each group of species will behave more or less the same within certain limitations. Thus, the species may be partially identified by the fermentation end products. The homofermentative species produce lactic acid as a major end product of growth, the amount varying from 85 to 95 per cent on the basis of the hexose sugar fermented. Small amounts of carbon dioxide and acetic acid are produced. Equimolecular amounts of lactic acid and acetic acid are produced from the pentose sugars. The heterofermentative species convert up to 50 per cent of the glucose utilized to lactic acid, 20 to 25 per cent to carbon dioxide, and 20 to 25 per cent to alcohol and acetic acid. Levulose is partly converted to mannitol, but, since mannitol is fermentable by many strains, it may be an intermediate rather than an end product of fermentation. Pentose sugars are fermented to lactic and acetic acids.

To those who have studied and compared the lactic acid rods and the lactic acid enterococcus group of cocci, the physiological grouping proposed by Orla-Jensen is logical. Furthermore, it seems desirable to confine the family to an exclusive and well defined family of two morphological tribes, the coccus forms and the rod forms.

The genus *Streptococcus* is without doubt the best known of the family. The relationships of species and strains have been more adequately treated than any of the other genera. Attention might be given particularly to the review by Sherman (114), the chapter by Topley and Wilson (133), as well as the classifi-

[2] Approved for publication by the Director of the New York State Agricultural Experiment Station as Journal Paper no. 900.

cation in *Bergey's Manual*, Breed *et al.* (16). Obviously, more work should be conducted to show the relationship among those classifications based upon fermentation reactions, those based upon changes produced in blood media, and those based upon antigenic structure. Studies to date are far in advance of any similar studies on the species of other genera in the family. Topley and Wilson believe that the separation of the genera *Diplococcus* and *Streptococcus* as proposed by Winslow *et al.* (144) is undesirable.

The species of the genus *Leuconostoc*, originally isolated by Cienkowski (23) as spoilage organisms in sugar factories, are heterofermentative. The sucrose fermenting strains usually produce a characteristic slime in sucrose media. Hucker and Pederson (58) reviewed and described the relationships among species within the genus and to the streptococci. Inclusion of only three species in the genus has been generally accepted. Previously, Orla-Jensen (94) had separated the genera, but had used the generic name *Betacoccus*. The earlier name *Leuconostoc* used by Van Tieghem (137) cannot be disregarded. Later studies with these organisms which may have taxonomic value are those of McClesky *et al.* (83), in which they grouped strains on the basis of type of slime produced, and that of Niven *et al.* (91), in which they described sucrose fermenting strains of *Leuconostoc* isolated from sausage that do not produce the characteristic slime. *Bacterium gracili* isolated from wine and described by Müller-Thurgau (87) may be of this type. Pederson and Ward (100) have described similar strains from fermenting cucumbers which later Pederson and Albury (unpublished) induced to form the typical slime by repeated transfer in sucrose media. At present it seems desirable to consider these as non slime-forming strains of *Leuconostoc mesenteroides*.

Pasteur (97) observed a lactic acid producing sarcina-like organism associated with a type of spoilage in beer. Balcke (3, 4) studied the spoilage, and he applied the binomial *Pediococcus cerevisiae* to the species. Since the relationship of this organism to other lactic acid producing types had not been clearly shown, thus they were included in the family Micrococcaceae with the type species *Pediococcus cerevisiae* Balcke in the sixth edition of *Bergey's Manual*. Shimwell (116) has reviewed the subject of beer disease organisms and concluded that the beer sarcina or pediococci should be accepted in the plant division of the genus *Streptococcus*. He did not regard them as true sarcina or micrococci. They are readily distinguished (99) from the species of the genus *Streptococcus* by their tetrad grouping and their comparatively high acid production. These morphological and physiological characters and the fact that they produce inactive lactic acid seem sufficient to separate them from the genera *Streptococcus*, *Micrococcus*, or *Sarcina*. They should be considered as a separate genus in the tribe Streptococceae of the family Lactobacteriaceae. There seems little justification for the use of more than one species name until further study may show more significant differences between the types described at present.

The genus *Lactobacillus* is a well defined group of lactic rods of two types, one homofermentative and the other heterofermentative. The generic name *Lactobacillus* was proposed by Beijerinck (9) to include all rod shaped organisms that produce levo-lactic acid. Although Beijerinck mentioned *Lactobacillus fermenti*

first, *Lactobacillus caucasicus* was designated as the type by Winslow *et al.* (144). The identity of the organism from kefir grains described and named *Dispora caucasica* by Kern (63) is confused. The organism he isolated in Cohn's solution was a spore-former. Without doubt, he had a mixed culture since he described this organism as varying in size and stated that the bipolar spores of *Dispora caucasica* did not distort the cell. However, the oval spores of the organisms isolated in Cohn's medium were described as larger than the rod. Since we know now that kefir grains contain a mixed culture of lactic acid bacteria, yeasts and spore-formers, there is little doubt that the spores he isolated were not the same as the bodies in the rods. These bodies were undoubtedly granules, such as are now known to be common in the long rod, lactic acid bacteria. Buchanan (19) says in regard to the generic name: "If Kern misinterpreted what he saw, and in reality named the high-acid bacillus of kefir *Dispora*, the name, in spite of its lack of appropriateness would appear to be valid." This species is now considered by Breed *et al.* (16) to be that of a prototype of *Lactobacillus caucasicus* Beijerinck. Beijerinck (8) was apparently the first to isolate a lactobacillus (*Bacillus caucasicus*) from kefir in pure culture and to give a sufficiently complete description to make re-indentification possible.

The *Bacillus caucasicus* of von Freudenreich (139) should not be confused with that of Beijerinck in that Beijerinck's culture was homofermentative whereas von Freudenreich's was heterofermentative. The latter was presumably the same as the *Betabacterium caucasicum* of Orla-Jensen (94).

Lehmann and Neumann (72) considered the generic name *Lactobacillus* unsuitable and suggested the name *Plocamobacterium* Löwi. They base this genus upon Doederlein's bacillus called *Bacillus vaginae* by Kruse, but the description given is that of *Bacillus crassus* Lipschütz (75), an organism isolated from the secretions of a diseased vagina. This organism, which is the only one placed in *Plocamobacterium* by Löwi, liquefies blood serum and should not be confused with Doederlein's bacillus, a typical acid producing organism isolated from the secretions of a healthy vagina. The latter is related to *Lactobacillus acidophilus*. Therefore, the genera *Lactobacillus* and *Plocamobacterium* should not be regarded as identical even though other species placed in the genus *Plocamobacterium* by Lehmann and Neumann are members of the genus *Lactobacillus*.

Caseobacterium was proposed by Orla-Jensen (93) and *Acidobacterium* by Schlirf (111) for the lactic acid producing rods. *Lactobacterium* was used by Van Steenberge (136) for a physiological group of lactic acid producing rods. Beijerinck suggested lactobacter as a trivial name.

Orla-Jensen (94) proposed the separation of these rods into three genera: the high acid producing, high temperature genus *Thermobacterium*; the high acid producing, lower temperature genus *Streptobacterium*; and the heterofermentative genus *Betabacterium*. He compared physiologically these groups of lactic rods with the genera of cocci *Streptococcus* and *Betacoccus* (*Leuconostoc*). Some think that the line of demarcation is somewhat more pronounced than among the lactic rods, in that species of the genus *Streptococcus* always produce *dextro*-lactic acid whereas species of the genus *Leuconostoc* always produce *levo*-lactic acid and,

in addition, usually form a dextranous slime from sucrose. However, one type of the gas-producing rods, *Bacterium vermiforme* Ward (140) produces a typical slime similar to that produced by species of *Leuconostoc*, and all strains of the genus *Leuconostoc* do not produce slime. In a recent paper, Coolidge (25) has indicated that a heterofermentative could change to a homofermentative type fermentation. If such change can be induced at will, further doubt exists about dividing the genus *Lactobacillus* into two genera.

Some question arises also regarding the validity of the generic names selected by Orla-Jensen. Fuhrmann (41) first used *Thermobacterium* for the *Termobacterium* of Zeidler (149). *Streptobacteria* and *Streptobacterium* were employed by Billroth (12) to designate a growth phase of *Coccobacterium septica*; and later also by Maggi (82), Billet (11) and Jacqué and Masay (59). *Betabacterium* is based upon the von Freudenreich (139) concept of the organism of kefir grains *Bacillus caucasicus* von Freudenreich, and, therefore, the same basic criticism, that there is no acceptable type species, could be applied to this genus name that is sometimes with less reason applied to *Lactobacillus*. Moreover, *Betabacterium* Orla-Jensen is a synonym of *Saccharobacillus* Van Laer (134) type species by monotypy *Saccharobacillus pastorianus* Van Laer. Van Laer considered the latter organism the same as the filiform bacillus of Pasteur in *Etudes sur la Biere* (97). He observed that it grew both anaerobically and aerobically and that it produced small quantities of carbon dioxide which was considered as evidence of intramolecular respiration. The organism produced inactive lactic acid, volatile acid, mostly acetic, and appreciable amounts of alcohol. Thus the organism must have been a heterofermentative strain.

The description is also given by Macé (78) and by Henneberg (53). Henneberg (54) gave a more complete description of a strain isolated from beer. Shimwell (115) used similar methods of study and isolated and described in more detail the long thin, slow growing rods from beer. His study showed that the organism may be identified. The growth characteristics are typical of the original isolations of many of the slow growing, acid and alcohol tolerant, heterofermentative rods from wines, tomato products, salad dressings and similar acid products.

The name *Saccharobacillus pastorianus* is validly published in a standard publication. The type species of this genus may be and has been identified. In any proposal to separate the heterofermentative from the homofermentative lactic acid rods, the generic name *Saccharobacillus* has priority for the heterofermentative lactic acid bacteria. The generic name *Betabacterium* is a synonym. Thus the generic name *Lactobacillus* would still be used for the homofermentative genus whereas *Saccharobacillus* would be used for the less known heterofermentative genus.

A large number of gram positive, acid producing cocci and rods differ in one way or another from the typical physiological or morphological pattern but must be considered in any discussion of the family Lactobacteriaceae. Whether they shall be included in the family will depend on whether a broad or narrow viewpoint is taken. If a broad attitude, then the characterization of the family, tribes, and genera must be emended. If the viewpoint is narrow and restricted, so as to

abide fully by the present characterization of the family, then where the lines of restriction are to be drawn must be determined. As will be pointed out, at times the distinctions may be fine. At present, certain strains, species and even a genus exist which are generally accepted as belonging in the family, but which do not conform in one way or another to the family characterization. For example, although motility is not a character associated with these organisms, motile streptococci have been described on several occasions, and more recently motile lactobacilli have been described, Cunningham and Smith (27), Harrison and Hansen (47), Hayes (unpublished). In this latter example, I confirmed the characterization of the strain as one of *Lactobacillus plantarum*, not realizing that it was motile. Liquefaction of gelatin is another exception, but *Streptococcus liquefaciens* has always been considered as a true species of *Streptococcus*. Division of cells in two planes is a morphological character supposedly not a character of the family, but it is a characteristic of the genus *Pediococcus*. Anyone who has studied the pediococci realizes their close relationship to the other lactic acid bacteria. In fact, as pointed out previously, Shimwell would place the species in the genus *Streptococcus*. Other exceptions might be cited, but the important point becomes—where shall the separation be drawn? This requires a discussion of some of the true lactic acid bacteria with respect to those closely related to them.

The various gram positive, acid producing bacteria isolated from intestinal contents have been a source of confusion. As indicated in recent papers (96, 141), there is still some disagreement in regard to the difference between *Lactobacillus acidophilus* Moro (86) and *Lactobacillus bifidus* Tissier (131). The several anaerobic types described by Distaso (32) and Debono (30) which Castellani and Chalmers (21) assigned to the genus *Bacteroides* were at first believed to be closely related to the bifidus type. Eggerth (35) concluded that these types differed sufficiently from the gram negative species of the genus *Bacteroides* to justify classifying them either in the genus *Lactobacillus* or in a separate genus. Recently, Barker and Haas (7) suggested the generic name *Butyribacterium* for the gram positive rods that produce acetic acid and butyric acid from sugars. The new genus is based on the culture called *Lactobacillus bifidus* Type II by Weiss and Rettger (141) and Lewis and Rettger (73). However, as pointed out by Pederson (98), not all of the anaerobic, acid forming types are butyric acid formers or high volatile acid producers. Possibly some of these acid producing strains that show marked activity toward nitrogen compounds may be related to the *Bacillus crassus* of Lipschütz (75), the organism that is the type species of the genus *Plocamobacterium*.

The exact relationship of these anaerobic types, including those described by Lipschütz (75), Biocca and Seppilli (13), and Johnson and Pollard (62), to the true lactic acid bacteria is not clear. Recently, Harrison and Hansen (46) have described anaerobic strains of the bifidus type from the cecal feces of turkeys. Another of their strains of lactobacilli is motile (47), but in other respects is identical with strains of *Lactobacillus plantarum*. The various anaerobic types similar in many ways to the typical lactobacilli but which show physiological gradations from the *Lactobacillus bifidus* strains through the various anaerobes,

some of proteolytic nature, and to the true butyric acid producers need much more study. The butyric acid producing strains can be distinguished at present only by means of a biochemical study of end products of growth. Similarly, the high volatile acid producing strains cannot be distinguished from those strains exhibiting the more usual physiology except by biochemical study. A series of generic names has been proposed by Prévot (105) for many of the species of anaerobic, gram positive, largely parasitic rods, but we know too little about them to discuss them intelligently.

Apparently there is a physiologically parallel group of anaerobic organisms with somewhat variable reactions which Prévot (104) has associated with the genus *Streptococcus*. One of these, *Streptococcus lanceolatus*, is described as producing butyric acid and other higher fatty acids and, therefore, may be comparable to the species *Butyribacterium rettgeri*. Obviously, it should not be included in the genus *Streptococcus*. As is true with the rod forms, the metabolic processes of these organisms must be studied further before they can be classified properly.

The organisms sometimes included in the genus *Leptotrichia* might possibly be considered as low acid producing lactobacilli insofar as present knowledge is concerned. The mouth strains studied by Thjøtta *et al.* (130) were identified as strains of *Leptotrichia buccalis* (Robin), Trevisan, but were differentiated from the lactobacilli on the basis of mannitol fermentation. The fermentation of mannitol is variable among the several species and strains in the genus *Lactobacillus*. However, the high final pH 4.73 to 5.17 observed for these strains by Thjøtta *et al.* is equivalent to less than 0.1 per cent acid which is considerably less acid than that ordinarily produced by normal smooth strains of lactobacilli. Rough strains of lactic acid rods ordinarily may produce less acid than smooth strains. It is possible that these mouth strains bear the same relationship to the high acid producing rods as do the parasitic streptococci to the high acid producing streptococci; or they may be intermediate between lactobacilli and the low acid producing proteolytic types in other genera. Sullivan *et al.* (129) divide the oral lactobacilli on the basis of acidity but do not consider amount of acid produced suitable as a basis for generic separation. Bibby and his coworker (10) suggest that the low acid producing, filamentous oral bacteria are strains of the species *Leptotrichia buccalis*. They demonstrated in later studies darkening of the teeth. An excellent review of the role played by lactobacilli in dental caries is presented by Rosebury (109). He emphasizes the heterogeneous nature of the group and thinks that there is no generally acceptable classification. The true status of these organisms as well as all others in the genus will remain unsettled until more comparable biochemical studies are conducted. Tittsler *et al.* (132) and Rogosa *et al.* (108) have made a start in this direction which should be extended further.

Orla-Jensen (93) proposed the genus *Propionibacterium* for organisms that convert lactates and lactose to propionic acid, and *Propionicoccus* (95) for the coccus type. In certain respects, *e.g.*, acid production, the need for complex media, anaerobic growth, and morphological characters, these organisms can be likened

to the true lactics. However, according to van Niel (135), they are catalase positive, have an oxidative system, and produce propionic and acetic acids, and carbon dioxide. The studies of Werkman and Wood (142) and Delwiche (31) show a type of metabolism quite different from that of the lactic group. The genus has some characteristics which suggest it should be included in the family Corynebacteriaceae; some species in this family produce propionic acid. Douglas and Gunter (33) suggest that the species *Corynebacterium acnes* should be placed in the genus *Propionibacterium*.

The genus *Microbacterium* was allied to the genus *Lactobacillus* by Orla-Jensen (94) although the name "Microbacterium" was first used as a tribe name by Cohn (24). The species in the genus are catalase positive and aerobic, and differ in other respects from the true lactic acid bacteria. Jensen (60) considered the genus as an intermediate group between the genus *Lactobacillus* and the acid producing, aerobic species of *Corynebacterium*.

It is obvious that there are border line species and strains related in one way or another to the true lactic acid bacteria. Many species have been described which cannot be included in classifications, not because of improper description, but rather because the descriptions are too incomplete to determine correct relationships. Possibly genera in the family Lactobacteriaceae are too exclusive and their border lines should be broadened. In general, it seems such action must wait until the newer methods of studying morphological, biochemical, nutritional and serological relationships show where excluded types may be placed.

Obviously, knowledge of end products of fermentation among these organisms is necessary for correct identification. Kluyver and van Niel (67) have emphasized this need, and the studies of Barker and Haas (7) have substantiated it. For example, without the analysis of fermentation endproducts, butyribacteria might well be confused with lactobacilli.

The classification and basis of a key for separation of the various species in the several genera are not entirely satisfactory. The correlation of the several methods of separating the species in the genus *Streptococcus* seems to have good possibilities. Species usually follow a general pattern in regard to sugars and carbon compounds utilized. However, because of the variations in the fermentation of such compounds, their use in a key often leads to confusion. In the genus *Lactobacillus*, they are used for want of better criteria. Some of the more recent work on growth requirements, Rogosa *et al.* (108) and Tittsler *et al.* (132) may be of value in characterizing species. The acceptance of new specific names in a classification such as presented in *Bergey's Manual* can lead to further chaos unless the organisms in their various growth phases are compared carefully with known species, and real reasons are furnished why the new type should be considered as a species. Variability in species is established, *e.g.*, the so-called rough strains differ in many respects from the smooth forms. This fact should be borne in mind when describing new types and applying new specific names. The rough strains, usually obtained under the more adverse growth conditions, tend toward production of fuzzy colonies, longer and thinner rods and chains of rods, more marked granulation, lower acid production, more anaerobic requirements for growth, inability to utilize some carbon compounds regularly utilized,

and general viability in laboratory media. When carried in the laboratory, the rough strains tend to change to the smooth type. Many of the anaerobic types, particularly those from the alimentary tract, may be representatives of growth phases which by further study may be shown to be typical lactic acid bacteria.

In brief summary, the family Lactobacteriaceae should include the genera *Streptococcus*, *Diplococcus*, *Leuconostoc*, *Pediococcus*, and *Lactobacillus*. If the genus *Lactobacillus* is to be divided into a homofermentative genus and a heterofermentative genus to correspond with the similar separation of the genera *Streptococcus* and *Leuconostoc*, the name *Saccharobacillus* should be applied to the heterofermentative type. The catalase positive genera, *Microbacterium* and *Propionibacterium*, the various anaerobic species and genera of rods and cocci, including the genus *Butyribacterium*, cannot be included in the family unless the characterization of the family is emended. The lines of demarcation of some of the anaerobic types from the typical lactic acid bacteria are not clear.

Part II. The Nutrition of the Lactic Acid Bacteria

ESMOND E. SNELL

All living organisms require for growth a utilizable form of energy, appropriate nitrogen and carbon containing materials to permit synthesis of the various components of their protoplasm, and certain inorganic salts. These nutritional requirements must be supplied in appropriate concentrations, and in a physical environment consonant with growth of the organism. Individual microorganisms vary tremendously in the complexity of the growth media necessary to satisfy their nutritional requirements. From the standpoint of these requirements, the lactic acid bacteria (including the genera *Lactobacillus*, *Streptococcus* and *Leuconostoc*) are among the most complex organisms so far investigated. A brief summary of their nutritional requirements follows. More detailed reviews, in which individual contributions to the subject are more fully documented, have appeared elsewhere (68, 94, 102, 120, 121).

ENERGY SOURCE

The soluble carbohydrates, *e.g.*, glucose, lactose, sucrose, are by far the most important compounds utilized as an energy source by lactic acid bacteria. Different species differ in their ability to ferment individual sugars, and this characteristic is important for their classification. The most commonly used (and the most universally available) carbohydrate for their culture is glucose, but species which prefer other sugars, *e.g.*, lactose or xylose (120), and even fail to ferment glucose readily have been described.

Individual species of lactic acid bacteria are designated as *homofermentative* or *heterofermentative*, depending upon their action on glucose. Homofermentative organisms (*e.g.*, *Lactobacillus casei*, *Lactobacillus bulgaricus*, *Lactobacillus plantarum*, *Streptococcus faecalis*) ferment up to 95 per cent of the utilized glucose, other hexoses, or fermentable disaccharides to lactic acid, as indicated by the equation:

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH$$

The remainder of the sugar is converted to carbon dioxide, traces of volatile acids, and cellular protoplasm. All current evidence indicates that lactic acid is formed by reactions of the well known Embden-Meyerhof scheme.

Heterofermentative organisms (*e.g.*, *Lactobacillus gayonii*, *Lactobacillus lycopersici*, *Leuconostoc mesenteroides*) also form lactic acid from hexoses, but produce in addition relatively large amounts of acetic acid, ethanol, carbon dioxide and other products. Both heterofermentative and homofermentative organisms characteristically ferment pentoses according to the equation:

$$C_5H_{10}O_5 \rightarrow CH_3CHOHCOOH + CH_3COOH$$

The mechanism of these fermentations is not well known but is being investigated (42, 45).

Some lactic acid bacteria grow with certain noncarbohydrate materials, *e.g.*, citric acid, as an energy source. The importance of such energy sources under natural conditions is probably small except under unusual circumstances. Amino acids and fatty acids do not serve as energy sources in laboratory media and presumably are inert in the natural habitat of these organisms.

NITROGEN AND CARBON CONTAINING COMPOUNDS—GROWTH FACTORS

Growth of all lactic acid bacteria tested is stimulated by addition of ammonium salts to media that are deficient in certain amino acids. This indicates that ammonia can serve as the source of nitrogen for the synthesis of nonessential amino acids and other nitrogenous compounds that are essential components of protoplasm, but are not supplied preformed in the medium. For other organisms, *e.g.*, *Lactobacillus helveticus*, ammonium salts stimulate growth even in highly complex media for reasons still unknown (113). Similarly, an external supply of carbon dioxide either stimulates, or is essential for, growth of several lactic acid bacteria when the availability of certain organic nutrients (*e.g.*, certain amino acids) is restricted (77, 121). Under such conditions, carbon dioxide is utilized in the synthesis of these compounds (71) and must be considered as an essential nutrient. Most of the combined nitrogen and carbon that appears in the cell, however, must be supplied to these organisms as preformed organic compounds, which for one reason or another cannot be synthesized by the cell. These various organic "growth factors" are listed together with representative organisms that require them, in tables 2.1–2.3, and discussed briefly in the following sections.

Vitamins and related growth factors. The vitamins and related growth factors required by the lactic acid bacteria are listed in table 2.1. As a group, they are all required in much smaller quantities than the remaining organic growth factors, even though major differences exist in the amounts of the individual vitamins required. Pantothenic acid is illustrative of a vitamin that is required in relatively large amounts for growth; 0.2–0.5 μg per 10 ml are required to permit maximum growth of the organisms listed under conditions so far employed. In contrast, the requirement for the more active vitamins, *e.g.*, vitamin B_{12}, is only 1/1,000 to 1/100 of this amount. The minute amounts of these compounds required reflect the catalytic role that they play in metabolism. Unlike the amino acids, fatty acids, etc., they are not major structural components of the cell.

TABLE 2.1

Vitamins and related growth factors required by lactic acid bacteria

GROWTH FACTOR	REPRESENTATIVE ORGANISMS REQUIRING THE COMPOUND FOR GROWTH	REFERENCES*
p-Aminobenzoic acid	*Lactobacillus plantarum* *Lactobacillus arabinosus*	120, 121, 123
Biotin	*Lactobacillus plantarum* *Leuconostoc mesenteroides* *Streptococcus faecalis*	120, 121, 123
Folic acid (Pteroylglutamic acid)	*Lactobacillus casei* *Streptococcus faecalis*	120, 121, 122
Folinic acid (Leucovorin, 5-Formyl-5,6,7,8-tetrahydropteroylglutamic acid)	*Leuconostoc citrovorum*†	103, 110, 122,
Lipoic acid (Thioctic acid, Acetate-replacing factor, Protogen)	*Lactobacillus casei*‡ *Streptococcus lactis*‡	66, 121, 147
Nicotinic acid	*Lactobacillus arabinosus* *Leuconostoc mesenteroides* *Streptococcus faecalis*	119, 120, 121, 122
Pantothenic acid	*Lactobacillus casei* *Leuconostoc mesenteroides* *Streptococcus faecalis*	119, 120, 121, 122
Pantetheine or Pantethine	*Lactobacillus acidophilus*§ *Lactobacillus bulgaricus*	26, 122, 124
Riboflavin	*Lactobacillus casei* *Streptococcus lactis*	119, 120, 121, 122
Thiamine	*Lactobacillus fermenti* *Streptococcus lactis*	120, 121, 122
Vitamin B_6‖		
Pyridoxal	*Lactobacillus casei*	119, 120, 121, 122
Pyridoxamine	*Streptococcus faecalis*	119, 120, 121, 122
Pyridoxamine phosphate	*Lactobacillus acidophilus*¶ *Lactobacillus delbrueckii*¶ *Lactobacillus lactis*¶	50, 84, 120, 122
Vitamin B_{12}	*Lactobacillus lactis* *Lactobacillus leichmannii*	18, 44, 107

* Where possible, references are to review articles that cover both historical and metabolic aspects of the requirement for these vitamins. With the newer growth factors, for which review articles are unavailable, articles with references to related papers and subjects have been selected.

† Very large amounts of folic acid (relative to the folinic acid requirement) permit growth in the absence of folinic acid.

‡ Growth responses to this vitamin are obtained only when these organisms are grown in the absence of acetate. Under these conditions, marked stimulation of growth occurs; however, growth eventually occurs in the absence of added growth factor.

§ Very large amounts of pantothenic acid (relative to the pantethine requirement) permit growth in the absence of pantethine.

‖ Pyridoxine, in very large amounts, promotes growth in place of pyridoxal or pyridoxamine, probably because it reacts with components of the medium to form these latter compounds in very small yield.

¶ Very large amounts of pyridoxal or pyridoxamine (relative to the amount of pyridoxamine phosphate required) permit growth in the absence of pyridoxamine phosphate.

The amount of a vitamin required by a given species is not, however, a constant. These substances are catalysts for many reactions of both the catabolic and anabolic phases of metabolism. Where a given vitamin is required for a synthetic reaction, it is sometimes possible to supply the product of this reaction to the cell in a utilizable form, thereby eliminating the necessity for the synthetic reaction. This circumstance may result in a lower requirement for the vitamin. Many illustrations of this have been discussed elsewhere (122, 123); a single instance will suffice here. The biotin requirement of *Lactobacillus arabinosus* is 10 times as high in a medium lacking aspartic acid as in a medium containing ample amounts of this amino acid. This suggested that biotin was in some way essential for synthesis of aspartic acid by this organism, and this has been confirmed by tracer experiments (71, 122).

TABLE 2.2

Amino acids required by lactic acid bacteria

L-Alanine	Glycine	L-Proline
D-Alanine	L-Histidine	L-Serine
L-Arginine	L-Isoleucine	L-Threonine
L-Aspartic acid	L-Leucine	L-Tryptophan
L-Cysteine	L-Lysine	L-Tyrosine
L-Glutamic acid	L-Phenylalanine	L-Valine

TABLE 2.3

Miscellaneous additional organic compounds that stimulate or are essential for growth of certain lactic acid bacteria

NUCLEIC ACID DERIVATIVES	FATTY ACIDS
Adenine	Acetic acid
Hypoxanthine	Oleic acid (or other higher unsaturated fatty acid)
Guanine (Guanylic acid)	
Uracil (Orotic acid, uridine)	DERIVED AMINO ACIDS
Thymine	L-Asparagine
Thymidine	L-Glutamine
Other desoxyribosides	Peptides

Different combinations of the growth factors listed in table 2.1 are required for different organisms (120, 121); no single species is known which requires all of the vitamins listed. Under defined conditions, the requirement for these compounds is highly constant and reproducible. The current availability of defined media in which such requirements can be determined readily suggests use of these characters in classification of the lactic acid bacteria.

Amino acids. The number and identity of the amino acids required by lactic acid bacteria are highly dependent upon the vitamins that are supplied in the medium. In media that are low in biotin, many of these organisms (*e.g.*, *L. arabinosus*, *S. faecalis*) require aspartic acid but grow without it when large amounts of biotin are supplied. Similarly, certain of these organisms (*e.g.*, *S. faecalis*) require serine for growth in media low in folic acid but not in media

richly supplied with this vitamin (122, 123). Vitamin B_6 is especially important in this connection. Some organisms (*e.g.*, *S. faecalis*) grow in media that are very low or lacking entirely in vitamin B_6 only if all of the amino acids listed in table 2.2 are supplied; each of these amino acids (including D-alanine) is required for growth under these conditions. When vitamin B_6 is supplied in excess, many of these amino acids are unnecessary for growth, *i.e.*, they are then synthesized by the test organisms and need not be supplied preformed in the medium.

These results reflect certain of the catalytic roles of the vitamins in metabolism. Biotin is concerned in a way not yet understood in aspartate synthesis, and folic acid in serine synthesis. Vitamin B_6 is required for synthesis of most of the amino acids; in this case, transamination frequently (but not always) is the catalytic step involved (57, 120, 122). The requirement for D-alanine for growth in the absence of vitamin B_6 is the only known instance where a D-amino acid is known to be *essential* for a living organism. In the presence of adequate vitamin B_6, this amino acid is synthesized and occurs in readily detectable amounts in the cell (122, 123). Many conflicting data concerning the amino acid requirements of lactic acid bacteria appear in the literature. These are primarily due to: (a) the use of different levels of the vitamins (especially vitamin B_6) in media; and (b) to contamination of certain commercially available amino acids with other amino acids.

With each of the vitamins in excess, lactic acid bacteria still require many different amino acids for growth, the exact number and combination being a characteristic of the organism examined. Some, such as *L. arabinosus*, will grow with eight to ten amino acids; others are much more exacting. *Leuconostoc mesenteroides*, for example, requires 17 of the 18 amino acids listed in table 2.2 for growth under these conditions. These requirements provide the basis for the microbiological determination of the amino acids, a subject that has been reviewed at frequent intervals (*e.g.*, 34, 112).

Miscellaneous organic growth factors. Space does not permit detailed consideration of the many additional organic compounds that either stimulate, or are essential for, growth of one or another organism of the lactic group. They are listed in table 2.3. Of these, adenine and guanine (purine bases) and uracil (pyrimidine base) are customarily added to synthetic media for assay of vitamins and amino acids and serve as precursors for synthesis of bacterial nucleic acids. The specificity of the requirement for these various compounds varies greatly from organism to organism. For example, some bacteria will utilize either uracil, orotic acid or uridine equally well; a strain of *L. bulgaricus*, however, requires orotic acid for growth and cannot use uracil in its stead.

The requirements for purine bases and thymine are intimately associated with those for *p*-aminobenzoic acid or folic acid; frequently, but not always, *either* the nucleic acid derivatives *or* the vitamins, but not both, are required (120, 122, 123). The desoxyribosides bear a similar relationship to vitamin B_{12}. However, some organisms, *e.g.*, a strain of *Lactobacillus delbrueckii*, require thymidine specifically and cannot grow when vitamin B_{12} is supplied in its stead (66, 84).

Acetic acid stimulates growth of most lactic acid bacteria. This nutritional

function of acetate is distinct from its role as a buffer, which becomes significant only at much higher concentrations. For some organisms, *e.g.*, *L. casei*, this effect is duplicated by very small quantities of lipoic acid, which functions catatically in the production of acetate from pyruvate (44, 107). For other organisms, *e.g.*, *L. arabinosus*, higher fatty acids and sterols partially replace acetate (44).

A somewhat similar relationship exists between biotin and unsaturated fatty acids (*e.g.*, oleic or linoleic acids). For a large group of organisms, including *Lactobacillus fermenti* and *L. arabinosus*, *either* biotin *or* the unsaturated fatty acid is required for growth; another large group of organisms, typified by *Lactobacillus acidophilus* and *L. bulgaricus*, requires the fatty acid specifically, and biotin does not replace it (120, 122, 123).

From these many examples of the interrelationships between nutrients, it is evident that a given bacterial species does not necessarily require a fixed and unchangeable assortment of growth factors, but that different combinations, both qualitatively and quantitatively, may suffice to permit growth by supplying the same nutritional deficiencies through different mechanisms.

Peptides. In media that contain free amino acids, the essential vitamins, and other essential nutrients, partial hydrolysates of proteins frequently increase the rate of growth and are sometimes even essential for growth of certain lactic acid bacteria. Usually, the identity of the stimulatory peptides as well as the mechanism by which they exert growth effects not permitted by their component amino acids is not known. A recent analysis of two such examples revealed two distinct mechanisms at work. In one, assimilation of a free amino acid (L-alanine), but not that of its peptides, was inhibited by another amino acid (D-alanine) present in the medium (65). The peptides hence promoted growth by supplying an essential amino acid in a utilizable form. In the second, a free essential amino acid (L-tyrosine), but not its peptides, was largely destroyed by the organism before it could be utilized for growth. The peptide supplied the essential amino acid in a form available for growth, but protected from destruction, and hence stimulated growth more effectively than did the free amino acid (64). Relationships similar to these undoubtedly exist between several other amino acids and their peptides and explain many of the instances of growth stimulation by peptides.

INORGANIC SALTS

Because of the many organic compounds required by lactic acid bacteria and the lack of techniques for freeing these of traces of inorganic ions, detailed knowledge of the requirements of these organisms for such ions is lacking. They are known, however, to require relatively large amounts of potassium, manganese, and phosphate; some also require magnesium (120). The requirement for a given ion is not always specific. For example, Rb^{+} replaces K^{+} completely for *S. faecalis*, though not for *L. mesenteroides*. Similarly, Mg^{++}, Sr^{++} and Ca^{++} seem partially to replace the Mn^{++} requirement of *L. arabinosus* (81). The general topic of inorganic nutrition of these and other organisms is one which needs a great deal more investigation.

CONCLUDING REMARKS

Study of the lactic acid bacteria has been extremely productive in enlarging our knowledge of nutrition and metabolism. Several of the vitamins (*e.g.*, pyridoxal, pyridoxamine, folinic acid, lipoic acid, pantethine) were discovered through such studies; others (*e.g.*, pantothenic acid, folic acid) were discovered independently and our knowledge of them greatly increased by use of these organisms. These bacteria have provided extremely useful tools for the determination of the vitamins and amino acids they require. The discussed nutritional interrelationships have provided several clues to previously unknown biochemical mechanisms.

The complexity of the nutrition of these organisms, indicated earlier, is apparent from this brief survey. This complexity in external requirements must reflect an internal simplicity in enzymatic constitution as compared with many other organisms that synthesize most of these compounds for themselves. Because of this simplicity, the lactic acid bacteria are extremely useful organisms for biochemical study of the reactions which they carry out, since many of the complicating byways of metabolism present in other organisms are lacking. It is now possible to grow most of these organisms either in entirely synthetic media or in media of largely known composition, and thus study in detail the influence of a given nutrient on the course of such biochemical reactions. The continued use of these organisms for such biochemical studies, now on its threshold, appears assured.

PART III. USE OF LACTIC ACID BACTERIA IN MICROBIOLOGICAL ASSAYS

DAVID HENDLIN

The isolation in recent years of a number of growth factors has led to a better understanding of the nutritional requirements of the lactic acid bacteria. The complex nutritional needs of these microorganisms for one or more of the B vitamins, purines, pyrimidines and an array of amino acids as well as their ability to dissimilate carbohydrates to an easily measurable end product, lactic acid, have made them an exceptionally useful group. They have found wide use in the estimation of the vitamin and amino acid content of various natural substances. In addition, several strains with requirements for unknown organic factors have proved to be invaluable research tools in the isolation of these substances. Such use has been amply demonstrated by the recent isolations of vitamin B_{12} and the citrovorum factor from liver.

The lactic acid bacteria, therefore, can be considered as an indispensable analytical tool to biochemical and nutritional research. Since an excellent review on the details of vitamin assays and their applications is given by Snell (121), this summary will deal with some of the newer techniques developed in recent years and the various factors which influence the response of lactic acid bacteria to vitamins and amino acids.

METHODS OF ASSAY

In general, assay procedures with lactic acid bacteria for vitamins and for amino acids are similar. Except for slight modifications in composition of medium, total volume and incubation temperature, little change has occurred in the past several years in the conventional tube assay procedure. This method, in brief, consists of using a medium devoid of the particular nutrient to be determined but which is otherwise nutritionally adequate. The medium is made double strength and then is diluted with an equal volume of sample containing a measurable quantity of vitamin. A dosage response curve is also run over a range of vitamin concentration which will give a graded response between no growth and maximal growth. After appropriate incubation, the quantity of growth is determined turbidimetrically or by titrating the lactic acid formed. By comparing growth responses of the known and unknown, the vitamin content of the sample can be calculated. Details of this procedure are described adequately elsewhere (121).

Two relatively new techniques, however, have been gaining wide usage during the last 5 to 10 years. These are the agar diffusion method and paper chromatography. Several papers have appeared which describe the successful use of an agar diffusion method for vitamin and amino acid determinations. The technique employed is a variation of the one in wide use for estimating antibiotic levels of fermentation broths and body fluids. The method consists of measuring "zones of exhibition" obtained by placing solutions of varying concentrations of the material being tested into small cylinders contained on petri plates. These plates have been flooded previously with seeded solid medium, deficient in the test substance. The diameters of the growth zones are proportional to the dosage of the test substance. Various modifications of this procedure have been devised, one of which utilizes paper discs in place of cylinders. Cup assays for thiamine, riboflavin, nicotinic acid, biotin, vitamin B_{12} and several amino acids have been described (1, 2, 39).

The cup procedure has the advantage of being easily adapted to routine handling of large numbers of samples with the least expenditure of time and effort. Furthermore, the necessity of adhering to a rigid aseptic regime other than in the preparation of inocula is eliminated. The cup-plate method, however, has the disadvantage of being significantly less sensitive than the tube method. In general, cup-plate methods require approximately 10 to 2,000 times the vitamin levels detectable by the conventional tube method and, therefore, cannot be used effectively in the assay of materials of low potency. The sensitivity of this assay appears to be a function of the molecular weight and diffusion characteristic of the particular growth factor.

Although in some cases differentiation of vitamin analogs and compounds metabolically related to them can be accomplished by the use of microorganisms with different sensitivities to these substances, this method cannot always be used successfully. Recently, paper strip chromatography has been applied to the separation and quantitative estimation of mixtures of various vitamins and their analogs. The technique consists of separating the vitamin analogs on paper by

chromatography and, after locating the active moieties by bioautography, leaching the active areas and assaying the eluates by standard tube procedures. In this manner, it has been possible to determine quantitatively the levels of vitamins B_{12} and B_{12a} in fermentation broths which contain mixtures of these two vitamin analogs (146). The vitamin B_6 complex has been separated similarly into its components, each of which then can be quantitatively estimated by microbiological assay (145).

METHODS OF EXTRACTION

Most amino acids and vitamins exist in nature as conjugates. Because these bound forms are not always readily available to microorganisms, extraction methods have been developed which are applied to samples prior to assay. Two general methods are in common use, namely, acid hydrolysis and enzymatic digestion. Hydrolysis, which is the simpler procedure, is used for releasing amino acids and those vitamins which are unaffected by acid treatment at high temperatures. In the past, hydrolysis was carried out with H_2SO_4, but more recently with HCl because of the lower toxicity of chloride for lactobacilli. After hydrolysis, samples are neutralized with NaOH. With some samples, especially those of low potency, the relatively high levels of NaCl resulting from neutralization become toxic to the assay organism. MacLeod and Snell (80) ascribed such toxicity to the competitive antagonism of potassium by sodium and suggested that pH adjustments be made with KOH.

Because of the instability of certain vitamins to acid hydrolysis, methods that employ enzyme digestion have been devised. Where the chemical nature of the complex is known, the choice of enzyme is relatively simple. Thus, in thiamine determinations, phosphorylytic enzymes are used to liberate the vitamin from cocarboxylase. With the other vitamins the choice of enzyme and incubation conditions for maximum release of activity are largely determined by trial and error. It was not surprising, therefore, to find that when some microbiological assays were compared with other methods, *i.e.*, chemical or animal assays, wide discrepancies occurred. For example, in the pantothenate assay, the papain-clarase method or the myalase P method yielded only 20 to 50 per cent of the expected values based on chick assay or β-alanine assay. The discovery of coenzyme A followed by the demonstration of its pantothenic acid moiety, resulted in the development of a quantitative extraction procedure for pantothenic acid by Lipmann and his collaborators (74).

With an increasing knowledge of the nature of vitamin complexes, extraction procedures become more quantitative and assay results less variable.

FACTORS AFFECTING MICROBIOLOGICAL ASSAYS

Temperature. Because the rate of growth, as well as the amount of growth, of microorganisms is influenced by incubation temperatures, it is not surprising that nutritional requirements, in many cases, are also markedly affected by temperature. Thus Price and Graves (106) observed that when incubator temperatures varied 3–4 C, the response of *Lactobacillus helveticus* to a given level

of riboflavin varied as much as 25 per cent. More recently, Borek and Waelsch (15) demonstrated that a difference in temperature of 2 C determined the essentiality of phenylalanine for *Lactobacillus arabinosus*. Under their experimental conditions, optimal growth of *L. arabinosus* was obtained at 35 C. No growth, however, was visible at 37 C unless phenylalanine was added to the medium.

Although the temperature directly affects growth responses of lactic acid bacteria to various growth factors, the use of proper air circulation or thermostatically controlled water baths has permitted adequate temperature control so that such fluctuations are well below the range of significance.

Hydrogen ion concentration. Few, if any, studies have been made on the effect of pH on the nutritional requirements of lactic acid bacteria. These organisms, however, have pH optima which are readily surpassed during growth. It becomes necessary, therefore, to buffer adequately the assay media so that the pH levels during growth are maintained within noninhibitory levels. Salts of acetic, phosphoric and citric acid are usually employed for this purpose. Since several lactic acid bacteria show requirements for acetate or an acetate-replacing factor, acetate is the buffer of choice in assay media (43). MacLeod and Snell (79) noted that the addition of one per cent citrate to the growth medium increased the manganese requirement of *L. arabinosus* approximately 20-fold. For this reason, when salts of citrate are used as buffers, it is necessary to increase the metal concentrations of the medium because of the strong chelating action of citrate.

Carbon dioxide. Although most lactic acid bacteria grow well in chemically defined media without added CO_2, several reports in the literature indicate that under certain conditions CO_2 plays an important role in the nutrition of some species. Thus, the requirements of *L. arabinosus* and *Streptococcus faecalis* for phenylalanine, histidine and aspartic acid are eliminated by 6 per cent CO_2 (77). We have observed an effect of CO_2 tension on the biotin requirement of *Lactobacillus lactis* Dorner (ATCC 10,697). In media containing half-maximal biotin, maximal growth is obtained when the incubation atmosphere contains as little as 1 per cent CO_2 (22).

Evidently an interrelationship exists between temperature, pH and CO_2 since both temperature and pH exert a direct effect upon available CO_2. Lowered pH levels and high temperatures are responsible for decreased CO_2 tensions. Little difficulty, however, is encountered with CO_2 fluctuation in the conventional tube assay. This circumstance probably arises largely from the rigid temperature control and buffering capacity of the medium in the assay procedure.

Oxidation-reduction potential. Because lactic acid bacteria are microaerophilic organisms, it is not surprising to find that they are somewhat inhibited by relatively low pO_2. Lowry and Bessey (76), in developing a microassay for riboflavin in which a 0.2 ml total volume was used, observed that the O_2 tension had an inhibitory effect on the assay organism. The use of a 10 ml total volume, however, combined with reduced conditions resulting from sugar breakdown products formed during sterilization, generally brings about favorable oxidation-reduction (O/R) potentials. Therefore, it has usually been considered unimportant to control the O/R potential of assay media. Literature reports, however, are

appearing in increasing numbers which implicate this factor as one of the causes of assay variations. Bohonos *et al.* (14) demonstrated that reduced conditions resulted in a marked increase in the pyridoxine requirement of *Lactobacillus casei*. Conversely, observations with *L. lactis* Dorner and *Lactobacillus leichmannii* indicate that the requirements of these species for vitamin B_{12} are markedly decreased under reduced conditions (52, 69, 70). By varying the oxygen tension of the assay media, it has been shown that *L. lactis* does not require vitamin B_{12} under strictly anaerobic conditions. Oxidation-reduction potential measurements indicated that the critical potential is in the range of 170 millivolts (70). Reducing agents such as ascorbic acid, thioglycollic acid, pyruvic acid and to a lesser extent glutathione all eliminate the requirement of *L. lactis* for added vitamin B_{12} if sufficient quantities are added to reduce the O/R potential to the critical level. In the vitamin B_{12} assay it is necessary to maintain a relatively high O_2 tension in order to elicit a response to vitamin B_{12}.

It has been our experience that the requirements of several other lactic acid bacteria for growth factors are also related to the O/R potential of the medium.

Growth-stimulating substances. Many lactic acid bacteria are known to require what are generally termed growth-stimulating substances. These compounds affect early growth by significantly shortening the lag phase. Such factors have been reported for *L. casei* (128), *L. lactis* (117), *L. leichmannii* (101, 118) and many other species. Skeggs and coworkers (118) reported that in the *L. leichmannii* vitamin B_{12} assay, nucleotides stimulated early growth above that obtained with vitamin B_{12} alone. Peeler *et al.* (101) noted that their strain of *L. leichmannii* required an unknown factor for optimal growth during overnight incubation. Such a requirement was responsible for assay discrepancies when liver preparations were tested for vitamin B_{12}. It is apparent from the nature of the response elicited that these substances play a major role in assay variation when overnight incubation is used. For this reason it is preferable to use assay procedures with longer periods of incubation (40 to 72 hours) when studying the vitamin content of crude substances. In this way the effect of stimulatory substances is completely eliminated. However, it is also possible to overcome stimulatory responses by either adding the substance in question or using large inocula of bacteria. The routine addition of enzymatic digests of casein to assay media is due in large part to their stimulatory effect on microorganisms. With the *L. leichmannii* factor, the use of a large inoculum shortens the lag phase considerably so that responses to the stimulatory factor are eliminated (101).

Metabolically related compounds. With many lactic acid bacteria there are some compounds which, though chemically unrelated to vitamins, are capable of replacing them for growth. Thus, *S. faecalis* is able to grow without pteroylglutamic acid in media supplemented with the pyrimidine, thymine (127). *L. helveticus*, *L. lactis*, and several other B_6-requiring organisms can grow without the B_6 complex provided D-alanine is supplied (50, 125). Various desoxyribosides support complete growth of *L. lactis* and *L. leichmannii* in B_{12}-deficient media (126, 148). Fortunately, the activity ratios between such compounds and their vitamin counterparts are extremely high so that, for the most part, simple dilu-

tion eliminates their response in routine assays. However, difficulty is encountered in estimating vitamin potencies of materials of low potency where dilutions cannot be made high enough to take advantage of high activity ratios. When this occurs, special procedures based upon some chemical differences or differential microbial assays are used.

Metabolically related compounds also manifest themselves in vitamin assays through their synergistic action. When such compounds are introduced into vitamin-supplemented media at ineffective concentrations, a growth response is obtained which is many times greater than would be expected from the vitamin alone. Bardos *et al.* (5) have noted that suboptimal levels of thymidine increased 10-fold the response of *Leuconostoc citrovorum* to folinic acid (5). We have observed a similar relationship between pteroylglutamic acid (PGA) and citrovorum factor. In the presence of 0.2 μg per ml of PGA, which in itself is ineffective in producing growth of *L. citrovorum*, the response to citrovorum factor was increased 3- to 4-fold (51). Similar results were also obtained with formylfolic acid (51).

Amino acid antagonisms. It has been recognized for some time that in many cases the essentiality of a given amino acid is related to the composition of the nutrient medium. Changes in amino acid concentrations frequently have resulted in inhibition of growth. Thus, Brickson *et al.* (17) demonstrated that the isoleucine requirement of *L. arabinosus* was dependent upon the leucine and valine content of the medium. When the concentrations of these two amino acids were low, the response to isoleucine was poor. They also observed that high concentrations of isoleucine in media supplemented with limiting levels of valine were toxic to *Leuconostoc mesenteroides*. In experiments with *L. lactis* we, too, noted that isoleucine as well as leucine and methionine was inhibitory, such inhibition being antagonized by increasing levels of valine (22). These observations emphasize the important role of amino acid antagonisms in microbiological assay procedures. It is evident that assay media should be composed of moderately high concentrations of amino acids so that little, if any, effect on composition of the medium results from the addition of assay samples. In so doing, assay drifts because of amino acid antagonisms are reduced to a minimum.

SUMMARY

The foregoing discussion emphasizes the complexity of microbiological assay procedures. It is evident that such assays are affected by a multiplicity of factors. Through careful studies on the interrelationship of nutritional requirements and such physico-chemical factors as pH, temperature, and O_2 and CO_2 tensions, it has been possible to decrease assay variation significantly so that ranges of precision well within limits of ± 15 per cent are obtainable.

With other assays relatively wide discrepancies are still evident. These undoubtedly arise primarily from inadequate extraction procedures and lack of sufficient knowledge of the nature of stimulatory factors. As these two assay variables are brought under control, many of the assays which at present show wide assay "drifts" will no doubt fall within the precision limits of many of the amino acid assays, namely ± 5 per cent.

PART IV. INDUSTRIAL SIGNIFICANCE OF THE LACTIC ACID BACTERIA

CHARLES F. NIVEN, JR.

With respect to their ability to grow under many different environmental conditions, the lactic acid bacteria are a versatile group of microorganisms. Some species are known to be relatively tolerant to high concentrations of sodium chloride, sugar, alcohol, or organic acids, such as acetic. Different species of the lactic acid bacteria grow over a wide pH range. Some are able to grow at pH 4.0 while others can initiate growth at pH 9.5. In the presence of sufficient fermentable sugars, some of the high acid producing strains lower the pH of their environment to below 3.0. The temperature range over which different species of these bacteria are able to grow is considerably wide. Some species develop well at temperatures slightly above the freezing point, while others have optimal and maximum growth temperatures approaching those of true thermophiles.

All lactic acid bacteria obtain energy for growth primarily by fermentative mechanisms. Therefore, they can grow well anaerobically. They are not putrefactive; few are proteolytic; and they are not strongly lipolytic. Usually, the flavor and aroma imparted by the metabolic products of these microorganisms are not considered to be objectionable. They are more likely to be considered pleasant, especially if acetoin is one of the fermentation products.

In spite of their fastidious nutritive requirements, the lactic acid bacteria are omnipresent in nature. Most vegetable and animal products contain adequate nutrients for supporting extensive growth of these microorganisms, especially if a fermentable carbohydrate is present. For these reasons, the lactic acid bacteria are one of the most important groups of microorganisms in the food and fermentation industries. In some instances, they serve many useful purposes, but their uncontrolled growth may result in serious economic losses in industry. In this review, a few examples of both desirable and undesirable fermentations by the lactic acid bacteria shall be discussed.

DESIRABLE FOOD FERMENTATIONS

The first use of lactic acid bacteria in the preparation of fermented foods has been lost in antiquity. Various fermented milk drinks, such as acidophilus and bulgarian milks, kumiss, kefir, leben, yogurt, and cultured skim milk, are familiar to us. Although they have largely lost their popularity as agents of longevity or as "cure-alls", some of these fermented milk drinks are becoming increasingly popular in this country as wholesome and palatable foods. Most of these fermented milk drinks are modern outgrowths of practices used as far back as history is recorded for preserving milk in a palatable form without refrigeration.

In the meat industry, the fermented sausages are counterparts to cheese in the dairy industry. There are many varieties of these sausages—their numbers perhaps rival the different kinds of cheese—but the dry, summer sausages are perhaps the most familiar. With respect to protein, fat, fermentable carbohydrate and salt content, these sausages are similar to cheese, and also, the principles of their manufacture and ripening or aging have many features in common with those employed in making cheese. As with cheese, the lactic acid

bacteria are definitely essential in the manufacture of these sausages. The desired tanginess and acid flavor noted in these meat foods are attributed to these bacteria, and the strong reducing conditions created by the bacteria are important in the production and maintenance of the rich, red color of these sausages. In their manufacture, most of these sausages are allowed to ferment at a relatively low temperature. Then they are subjected to a long "smoke" at a temperature favorable for the growth of lactic acid bacteria and, depending upon the variety, may be allowed to age for a period of time in rooms where the temperature and humidity are rigidly controlled.

In spite of their similarity, however, there are a few basic differences between the manufacture of fermented sausages and cheeses. For example, a fermentable sugar, usually dextrose or sucrose, must be added to the comminuted meats. Also, the meat pigment, myoglobin, must be chemically changed (fixed) by reaction with a nitrite salt to obtain the desired red color (nitric oxide myoglobin and nitric oxide hemochromogen). In contrast with the mixed cure (containing both nitrate and nitrite) customarily employed for the more common cured meat foods, a straight nitrate cure is usually used in the manufacture of fermented sausages. Therefore, dependence upon a nitrate reducing microorganisms is necessary for proper color fixation. It can then be appreciated that a delicate balance exists in the microbial flora of the fermenting sausage mix. If insufficient nitrate is reduced, improper color will result, but if too much nitrite accumulates, the desired growth and fermentation by the lactic acid bacteria may be impaired. It is not surprising, therefore, that failures occur occasionally.

Chance contamination and growth of the correct proportions of both the lactic acid bacteria and the nitrate reducing bacteria are relied upon in the manufacture of fermented sausages. One wonders why pure culture starters are not employed as they are in the cheese industry. Although this has been proposed for a number of years, and a patent has been issued for such a process (61), thus far, however, it has been difficult to improve the quality of these sausages with such starters. It seems that more research is needed to investigate the possible use of mixed culture starters that would also include nitrate reducing microorganisms.

To the question of whether the lactic acid bacteria play any beneficial role in the curing of our more common meat items, such as hams, frankfurters, bologna, the answer apparently is that no bacteria are necessary in the streamlined practices now generally employed in this country for curing most of our meat products. But lactic acid bacteria and other bacteria probably play an important role in the development of characteristic flavors in certain types of long-cured hams and other meats, which are still receiving favorable acceptance in some sections of the United States.

The baking industry has used lactic acid bacteria in the production of rye bread (38). The original rye bread, made entirely from rye flour, is based upon the development of progressive sour doughs over a period of 48 hours. Either a starter culture or a small portion of a previous batch of sour dough is employed

to initiate the desirable fermentation. Such rye breads are popular in the European countries; but because of their inferior texture and the long fermentation times required, not many of the so-called rye breads are now manufactured in the United States by the sour dough method.

For improving flavor, texture, color, and preservative qualities, the lactic acid bacteria are employed in the manufacture of a number of fermented vegetables of which sauerkraut and many varieties of pickles are examples. Most of these fermentations depend upon the growth of salt-tolerant, high acid producing *Lactobacillus* and *Leuconostoc* species that occur naturally on the vegetable. Little or no improvement is achieved by the use of pure culture starters. The development of the desirable bacterial flora is controlled largely by maintaining the proper temperature and salt concentration. Excessive salt tends to inhibit growth of the lactic acid bacteria and to allow undesirable fermentations to occur.

OTHER IMPORTANT LACTIC FERMENTATIONS

Lactic acid itself has many industrial uses and, therefore, its production has become important. The high acid producing, homofermentative lactic acid bacteria (*e.g.*, *Lactobacillus delbrueckii*, *Lactobacillus leichmannii* and *Lactobacillus bulgaricus*) have been employed for the commercial production of lactic acid.

The lactic acid bacteria are used also in the distilling industry; for example, frequently the final mash in which the seed yeast is grown is first inoculated with *L. delbrueckii* and incubated at 50–52 C until the desired pH is attained. Then, the culture is killed by heat; the mash is cooled and finally inoculated with the selected strain of yeast. It is said that such prefermentation of the medium improves the environment for yeast growth, and at the same time growth of other contaminating microorganisms is reduced to a minimum. In the production of whiskey by the sour mash method, this principle of biological pH adjustment also may be extended to the large fermentation tanks, but in a slightly different manner. In the fermentation of the mash, various *Lactobacillus* species invariably grow in large numbers toward the end of the three-day fermentation period. These lactic acid bacteria are introduced primarily as accidental contaminants, and the pH of the fermented mash is usually lowered to nearly 4.0. Whether these contaminating bacteria impart any desirable flavors and aromas to the finished whiskey is open to question. However, after the alcohol has been distilled off, part of the stillage residue is mixed with the fresh batch of mash to be fermented at the rate of approximately 25 per cent of the total volume. This process of "back-slopping" is a convenient and economical method of adjusting the mash to the desired initial acidity (approximately pH 4.5 for sour mash fermentations) and is believed to aid in the control of other contaminating microorganisms while creating an improved environment for the alcohol yeast.

The principle of biological pH adjustment also appears to be an important factor in the successful manufacture of soda crackers (85). Apparently, accidental contamination of the cracker dough with the lactic acid bacteria and their subsequent growth is depended on to prevent excessive increases in the pH of the

crackers as they are baked. If the pH rises above 8.5 during baking, the crackers are likely to be soapy or mealy, high in density, and tend to brown or scorch unusually fast.

UNDESIRABLE LACTIC FERMENTATIONS

Although the lactic acid bacteria serve many useful purposes in the food and fermentation industries, they can also be a nuisance and result in serious economic losses. Their presence in many instances, however, serves as an excellent index of the sanitary and storage conditions under which the particular food item has been handled. Just as milk will sour if it is not refrigerated properly, so will other foods if they contain fermentable sugars, as is commonly observed in certain meat foods such as bologna. Usually, the common microorganisms involved are various species of the lactic acid bacteria.

The frozen concentrated orange juice industry has suffered very heavy losses of their product because of the growth of lactic acid bacteria in the juice as it is being concentrated. According to Hays (48), some of the organisms encountered in this spoilage are *Lactobacillus brevis*, *Lactobacillus plantarum* var. *mobilis*, and certain *Leuconostoc* species. A typical buttermilk odor is produced in the concentrated juice; this off-odor appears to result from production of diacetyl from citric acid by the contaminating bacteria.

Although diacetyl may impart a pleasant aroma to dairy products, it may not be so pleasant in other foods or beverages. The honey-like flavor sometimes noted in spoiled beer appears to be due in part to this compound. This spoilage is often called "*Sarcina* sickness" and results from the growth of certain lactic acid bacteria (*e.g.*, *Pediococcus cerevisiae* (99) or *Streptococcus damnosus*) in the fermented beverage. Other lactic acid bacteria, especially *Lactobacillus pastorianus*, are able to sour beer. The low pH, low temperature and anaerobic conditions of fermentation and storage, and the presence of alcohol and the hop antiseptics in beer limit the kinds of spoilage microorganisms that are able to grow in this beverage. The lactic acid bacteria just mentioned, however, can grow well under these environmental conditions.

Not only is souring and off-flavor production by the lactic acid bacteria important in many of our foods and beverages, but silky turbidity is also a problem. This type of turbidity, indicative of extensive microbial growth, accompanies the beer spoilage already described. Wines likewise are subject to such "diseases" as was well recognized and successfully controlled by Pasteur. These wine diseases are commonly caused by *Lactobacillus hilgardii* and other heterofermentative lactobacilli (138) that belong to Pederson's "inactive" group. Fornachon, Douglas and Vaughn (37) described and named another species of this group, *Lactobacillus trichodes*, that is found particularly in appetizer and dessert wines. This microorganism tolerates as much as 20 per cent alcohol. It is difficult to culture in ordinary laboratory media.

The development of turbidity is also an important spoilage problem in many vinegar pickled foods. Experience has taught us that to prevent the growth of the acid-tolerant lactic acid bacteria in such foods a minimum concentration of

3.6 per cent acetic acid must remain in the aqueous phase of the food after equilibrium has been established (20). Such high concentrations of acetic acid tend to reduce the palatability of such foods. To prevent the growth of acid-tolerant lactic acid bacteria in these products at lower concentrations of vinegar, it is necessary to pasteurize the product or to add an acceptable chemical preservative.

Certain meat food specialties, such as vinegar pickled sausages, are subject to spoilage by the acid-tolerant lactobacilli. The fermentable sugars and other nutrients that leach out of the sausages provide an excellent medium for these microorganisms. On the other hand, pickled pigs feet can be preserved with considerably lower concentrations of acetic acid than 3.6 per cent. In their manufacture, the pigs feet are cooked in brine for long periods of time and finally rinsed thoroughly in flowing water. All fermentable sugars and other energy yielding substances are leached out of the tissues, and although this food may be contaminated as it is packed into the jars, the microorganisms are unable to grow because of an energy starvation.

As mentioned earlier, the lactic acid bacteria characteristically obtain energy for growth by fermentation rather than by oxidation. This does not mean, however, that these bacteria are incapable of oxidizing certain substances under aerobic conditions. Some members of this group oxidize such substrates as lactic, pyruvic, butyric, α-ketobutyric and valeric acids as well as a number of alcohols, including glycerol. Also, certain unidentified substances in meat juices are oxidized by some of these bacteria. Apparently, most or all of the energy released in these oxidative systems is lost and is not made available to the organism for growth.

Some of the so-called "oxidative" types of lactic acid bacteria are of considerable economic importance in the meat industry because they can alter the color of the cured meat pigment (nitric oxide hemochromogen) to a pale green when they grow on cured meat products. These microorganisms are peculiarly adapted to developing on such cured meat products as hams, frankfurters, or bologna. They are relatively salt tolerant, can grow at temperatures as low as 3 C, and they are able to oxidize unknown substances in the sausage with the production of hydrogen peroxide. Since all tissue catalase is destroyed in the curing and processing of the meats, the peroxide that is formed accumulates in sufficient concentrations to react chemically with the cured pigments. It appears that a series of oxidized porphyrin compounds is produced, one of which is greenish in color.

Taxonomically, the majority of the bacteria that discolor cured meats belong to a *Leuconostoc* species and a heterofermentative *Lactobacillus* species (91), each of which represents a new species within its respective genus. The *Lactobacillus* species which is more commonly encountered in natural outbreaks is rather fastidious (36) and would seem to be a representative of Pederson's "inactive" group. It is a low acid producer, and some strains are remarkably heat resistant (89).

Depending upon the stage of manufacture of the cured meat foods in which contamination and growth occur, there may be several manifestations of the

discoloration caused by these bacteria. For example, contamination and growth on the surface of the finished product may result in greenish patches of irregular size and shape developing on the cured meat surface. On the other hand, if the processing temperatures are insufficient to kill all of these bacteria in the interior of the larger sausages, then the cores may discolor on exposure to oxygen.

Almost any food in which sucrose is present is subject to spoilage owing to slimy or ropy development. The sugar refining industry has had to cope with this problem for a long time, and the meat industry has had similar experience. No doubt, in many instances the development of ropy curing pickle was due to the growth of slime producing lactic acid bacteria. Improved refrigeration, sanitation, and streamlined processing methods have virtually eliminated the occurrence of this condition in most meat curing cellars. That it has not been completely eliminated, however, is evidenced by a recent publication by Mundt and Pryor (88), in which they describe this condition in ham curing cellars in certain areas in the South. The condition could be alleviated merely by replacing the sucrose with dextrose in the curing formula.

Although the ability of *Leuconostoc mesenteroides* and *Leuconostoc dextranicum* to produce slime from sucrose is well known, this characteristic is not limited to these two species. Many different streptococci and lactobacilli have the property of synthesizing a dextran, and sometimes a levan, from sucrose.

Some industrial fermentation industries contend with lactic acid bacteria as undesirable contaminants. For example, these microorganisms are one of the most serious contaminants in the acetone-butanol fermentation industry. Such high acid producing species as *Lactobacillus leichmannii* may drastically reduce the yields of the solvents by lowering the pH of the mash to a point which is unfavorable for butanol production (40). Similarly, the distilling industry may experience reduced alcohol yields as the result of early and extensive growth of the high acid producing lactobacilli. It is said that these decreased yields are caused not only from competition for the fermentable substrate, but also from the inactivation of the residual α-amylase in the fermenting mash by the resulting low pH before all of the carbohydrates are converted to fermentable sugars.

PUBLIC HEALTH ASPECTS

In discussing the lactic acid bacteria, we should not neglect a few words concerning their significance for health. None of the *Lactobacillus* or *Leuconostoc* species is known to be pathogenic or to cause food poisoning in man. A few *Streptococcus* species, especially *Streptococcus pyogenes*, are human pathogens. Food-borne epidemics caused by these bacteria, however, have become a rarity because of modern sanitary methods of handling food.

One group of streptococci, namely the enterococci, may have some significance in the food industries. This group is receiving increasing attention by the public health authorities when it is found in foods. As the organisms are considered to be intestinal in origin, some authorities consider their presence in foods as being an index of pollution. The enterococci are a hardy group of bacteria and are capable of growing in foods at a rapid rate over a rather wide temperature range. They commonly occur in certain types of cheeses and other foods in large num-

bers and are believed by some to contribute toward the desirable flavor of these foods (29). In spite of their ubiquity in some of our common foods, as well as in the intestine, there is some evidence that they may cause food poisoning. Controlled human feeding tests with representatives of this group, however, have thus far failed either to incriminate or free these organisms of any suspicion of being food poisoning microorganisms (28). Because of their common occurrence, many food poisoning outbreaks have no doubt been blamed upon these organisms, while the real culprit escaped undetected.

A few years ago in connection with some bacteriological studies on a number of soured bologna samples that had spoiled because of underprocessing, an

TABLE 4.1

Similarity of the Streptococcus sp. isolated from bologna to Streptococcus faecalis

	STREPTOCOCCUS FAECALIS	UNIDENTIFIED STREPTOCOCUS
Growth:		
10 C	+	+
45 C	+	+
6.5% NaCl	+	+
40% Bile	+	+
pH 9.6	+	+
Survives 60 C, 30 min.	+	+
CO_2 from glucose	−	−
Lactic acid from glucose	L(+)	L(+)

TABLE 4.2

Contrasting characteristics of the bologna Streptococcus sp. and Streptococcus faecalis

	STREPTOCOCCUS FAECALIS	UNIDENTIFIED STREPTOCOCCUS
Growth: 0.3 units penicillin/ml	+	−
Decarboxylates tyrosine	+	−
Lancefield group D	+	*
Ferments higher alcohols	±	−
Curdles milk	±	−

* All of these streptococci belong to one serological type. Among a large collection tested, no enterococci reacted with this type-specific serum. No group D serum was available at the time of this study.

interesting group of lactic acid organisms was detected (90). Since some doubt existed as to their morphology, difficulty was experienced in placing them into their proper genus. Old cells tended to occur singly or in pairs and were ellipsoidal in shape, but young cells appeared to be true streptococci. Therefore, they have been placed tentatively in the genus *Streptococcus*. Study of the physiological characteristics of these bacteria revealed a striking similarity between them and the enterococci. None of the common physiological tests that might be employed served to differentiate them from the enterococci (table 4.1). However, additional tests performed on these bacteria showed that they were clearly different from the enterococci (table 4.2). Therefore, these bacteria definitely may be a source

of confusion when a food is being examined for enterococci. The habitat of these bacteria is unknown, but if they were of fecal origin, they probably would have been recognized long ago.

PROMISING INDUSTRIAL APPLICATIONS

We have already mentioned that many of the lactic acid bacteria synthesize high yields of polysaccharide from sucrose. The recent discovery that these polysaccharides, when partially hydrolyzed, are effective plasma substitutes opens a new industry that promises to grow to huge proportions. No doubt many other industrial uses for these polysaccharides will be developed. Those engaged in these studies should keep in mind that the types of microorganisms that synthesize these polysaccharides are not limited to the genus *Leuconostoc*. The polysaccharides synthesized by the different bacteria may differ not only in chemical structure but also in physical properties. Some are produced only under anaerobic conditions (49, 92). The selection of proper bacterial strains may reduce or eliminate the chemical manipulation of the isolated polysaccharide necessary to make it suitable for its intended industrial use.

A few of the streptococci and lactobacilli have been reported to synthesize powerful antibiotic substances (6, 56, 143). Perhaps these may never compete with penicillin or some of the other popular antibiotics in human therapy, but the possible employment of such bacteria in foods as starter cultures should stimulate our imagination as to possible means of controlling certain types of spoilages (55) as well as food poisoning hazards.

In conclusion, we must confess our gross lack of knowledge concerning the different types of lactic acid bacteria that normally occur in our foods. More study of these microorganisms is warranted, especially in the light of their possible useful applications. Employment of the lactic acid bacteria in the development of new, safe and appealing foods having good keeping qualities holds as much promise today as it ever has. The actual and potential usefulness of these microorganisms greatly overshadows their economic importance as spoilage bacteria.

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